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13. ABSTRACT *(Maximum 200 words)*

Telomeres are specialized tandemly repeated DNA structures found at the natural ends of most eukaryotic chromosomes. They are required both for protecting chromosome from degradation and replicating the ends of chromosomes. Most eukaryotes replicate telomere by a special reverse transcriptase called telomerase. Another pathway of telomere formation is telomere-telomere recombination that uses homologs either in telomeric or subtelomeric repeats as the substrates. I am working on two projects involving *Saccharomyces cerevisiae* telomere formations. *S. cerevisiae* replicate their telomeres by using the telomerase pathway. In the first project I focus on understanding the nature of the *S. cerevisiae* telomerase. I found the Est2p reverse transcriptase and RNA subunit *TLC1* form a 20S telomerase complex in vivo. In the second project I tagged the subtelomere Y' element with a marker to study telomere-telomere recombination. I found that telomerase minus cells can survive through telomere-telomere recombination using either TG-rich or Y' sequences as substrates.

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Shu-Chun Teng 9.3.98

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Table of contents

Introduction

Telomeres are specialized tandemly repeated DNA structures found at the natural ends of most eukaryotic chromosomes. They are required both for protecting chromosome from degradation and replicating the ends of chromosomes. Most eukaryotes replicate telomeres by a special reverse transcriptase called telomerase. This specialized enzyme uses a small CA-rich stretch in the RNA component as a template and the 3' hydroxyl group at chromosomal ends as a primer to extend GT-rich telomeric repeats. Another pathway of telomere formation is telomere-telomere recombination which uses homologs either in telomeric or subtelomeric repeats as substrates. In the fruit fly *Drosophilia*, telomeres contain multiple retrotransposons and elongate telomeres through transpositions (Zakian, 1995).

In my original proposal I planned to use a genetic screening to look for the reverse transcriptase component of yeast telomerase, but because it was cloned right before I joined Ginger Zakian's lab (Lingner et al, 1997), I began working on two projects involving *Saccharomyces cerevisiae* telomere formations. *S. cerevisiae* replicate their telomeres by using the reverse transcriptase Est2p and RNA subunit *TLC1*, which together form a telomerase complex. In the first project I focus on understanding the nature of the *S. cerevisiae* telomerase. I will describe my findings and future directions. In the second project I tagged the subtelomere Y' element with a marker to study telomere-telomere recombination. I found that telomerase minus cells can survive through telomere-telomere recombination using either TG-rich or Y' sequences as substrates. I am writing a paper to report this finding now.

Experimental methods

All the *E.coli* and yeast operations, RT-PCR, and Southern blot analysis were performed according to "Current protocols in Molecular Biology" (Ausubel et al, 1997).

Assumptions, procedures, results and discussion

Project I. *In vitro* study of Est2p, the catalytic subunit of telomerase

To study Est2p in vitro, first I had to trace proteins in cell extract. I tagged the full-length EST2 with GST protein under a *GAL1* promoter in a 2-micron plasmid. Proteins were over-expressed by galactose induction and whole cell extracts were incubated with glutathione sepharose beads. After washing off unbound proteins, precipitates were subjected to RT-PCR with primers amplifying *TLC1* and *RAP1* RNAs. While no amplified RAP1 from any pull-down products and no amplified TLC1product from GST alone and two other proteins, CDC13p and Est1p-BP, studied in our lab, an obvious signal was amplified from GST-EST2 pull-down products (figure 1). This result suggested Est2p and *TLC1* RNA associate with each other in vivo.

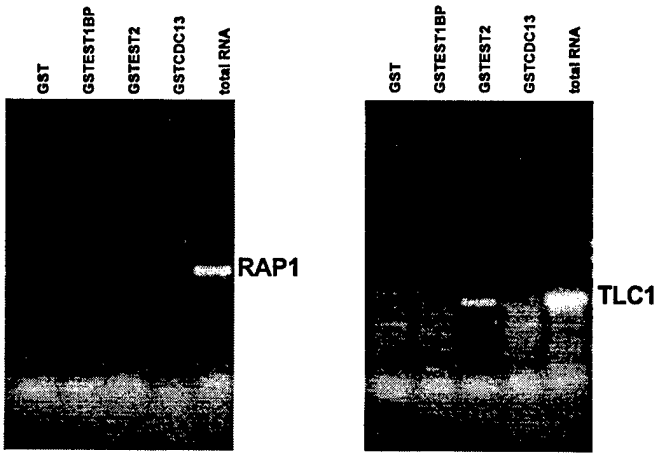

Figure 1. RT-PCR amplification of the TLC1 RNA from the GST-Est2p pull down.

The next question that I wanted to clarify is what is the size of telomerase. Knowing its size is important in prediction whether there are other components in the telomerase complex. To answer this question, S100 from whole cell extract were separated on a 15%-40% glycerol gradient. Fractions were collected and analyzed by RT-PCR with primers amplifying the *TLC1* RNA. PCR products were separated on a 2% agarose gel. The peak of *TLC1* was observed at the 20s position (figure 2). This result might suggest that there are other components in the telomerase complex.

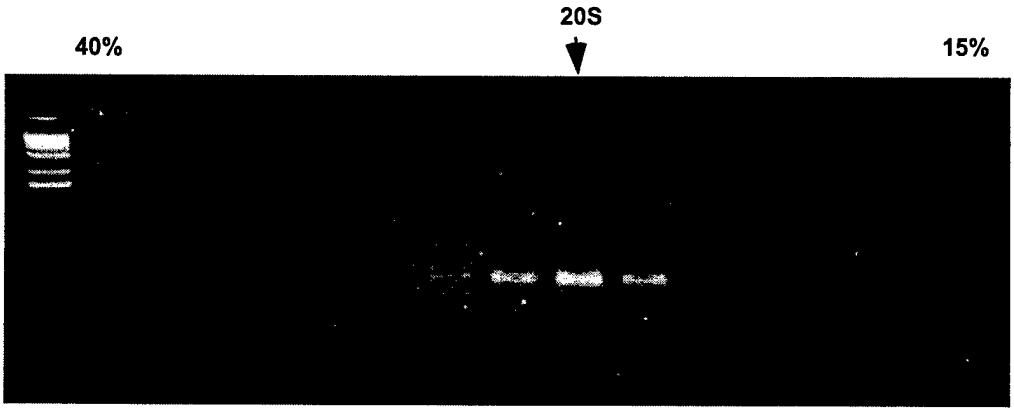

Figure 2. Glycerol gradient separation of the telomerase complex.

Telomerase may form a 20s complex in vivo when it does not bind to a telomere. However, the architecture of the complex when bound to the telomere remains elusive. I have been trying to answer this question by using an in vivo chromatin immuno-precipitation assay (ChIP). This assay uses formaldehyde as a cross-linking agent, because it reacts with amino and imino groups of proteins and of DNA within minutes. After mechanic force breakage, chromatin fragments containing Est2p can be immuno-

precipitated. Cross-links can be reversed and chromatin fragments can be PCR amplified (Aparicio et al, 1997).

Using this assay, I have readily immuno-precipitated Rap1p, a telomere binding protein with telomerase. An origin binding protein in the ORC complex can also be immuno-percipitated easily and shown increased signal to bind ARS at the early S-phase (Aparicio et al, 1997). A triple HA tag right before the stop codon of the chromosomal copy of *EST2* showed a signal barely above the background. Recently I constructed an *EST2* with nine copies of Myc tag on both the chromosomal copy and a 2 micron plasmid. ChIPs of these samples are in progress.

Project II. Telomere- telomere recombination suppresses loss of telomerase

S. cerevisiae replicate their telomeres predominately by the telomerase-dependent pathway using the Est2p and RNA subunit *TLC1* to form a telomerase complex. In the *tlc1* strain, cells lose telomeres gradually and most cells die within 100 generations. However, survivors come out (Singer and Gottschling, 1994). On the Southern blots, survivors showed two distinct patterns, which is similar to Lundblad's observation in the *est1* strain (Lundblad and Blackburn, 1993). On type extends telomeres by amplifying the subtelomeric Y' elements through recombination (I called it type I). The other type contains an irregular pattern of bands. Since these bands could be hybridized by Y'-3' end probe but not other Y' regions, Lundblad and Blackburn reasoned this type of survivors is similar to type I but uses the Y'-3' end as a substrate for recombination (we called it type II) (figure 3). Given there is no obvious $TG_{1\text{-}3}$ sequences at the Y' 3'end between the *Xho*I site and the telomere, I assume either the Y'-3' end is not the right substrate, or that there is a novel mechanism to replicate telomeres of type II survivors.

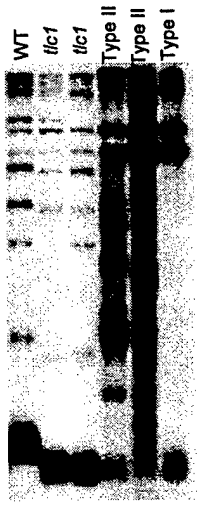

Figure 3. Southern blot analysis of *tlc1* survivors.

What known mechanisms can generate tandem repeats of mini-Y'? The most likely possibilities are transposition and nonhomologous end joining. The structure of Y' is similar to a retrotransposon. Retrotransposons are known to transpose at *Drosophilia* telomeres. Gabriel and Teng also found that retrotransposons have the ability to repair chromosomal breaks (Teng et al, 1996). I first tagged a single telomere with the *his3AI* marker and checked the histidine prototroph formation through type II recombinant formation. I observe no increase of histidine prototroph formation in *tlc1* survivors. The frequency is ~ $1x10^{-10}$ which is similar to tagging the *his3AI* marker at other place in the genome (data not shown).

Ku proteins were demonstrated to affect telomere formation (Gravel et al, 1998). To test if type II survivors replicate telomeres through nonhomologous end joining, I constructed a *tlc1 rad52* strain containing a CEN plasmid with *RAD52* and the *URA3* marker. After cells were senescenced and type II survivors were recovered, the *RAD52* plasmid was lost by the FOA selection. After four re-streakings, no cell could survive.

From the previous two experiments I ruled out the possibilities of transposition and nonhomologous end joining. These results strongly suggest that telomeres of type II survivors replicate and maintain themselves through the *RAD52*-dependent homologous recombination. The substrate for homologous recombination cannot be the Y' 3' end since there is no repetitive sequences between the *Xho*I site and TG_{1-3} for homologous recombination. The substrate must be TG_{1-3} or other unknown sequences. To understand the type II recombination in detail, I traced a single telomere by studying the *his3AI* tagged telomeres. 24 type II survivors were recovered and genomic DNA were collected, digested with *Xho*I and separated on a 0.9% agarose gel. Southern blot analysis was performed with Y'-5' end, Y'-middle region, Y'-3' end, X, rDNA, LTR, $C_{1-3}A$ and *his3AI*. As Lundblad and Blackburn reported, both Y'-3', $C_{1-3}A$, and his3AI probes can pick up multiple irregular size bands. Other probes could not hybridize these bands (data not shown).

To determine the sequence of these newly added telomeres, an inverse PCR stratagem was used (figure 4). Genomic DNAs of wild-type and two type II survivors were cut with *Xho*I. All *Xho*I digested fragments were blunted by Klenow fragment and four nucleotides. Self-ligated fragments were subjected to PCR using primer 1 and primer 2 facing opposite to each other. Expected sizes of PCR products from both wide-type and two type II survivors were obtained. These PCR products can be hybridized by $C_{1-3}A$ probe. Sequences of the PCR products by primer 3 showed only T and G signals, whereas primer 4 showed only A and C signals on gels. Since PCR products are population heterogeneous in sequence, I tried to clone them into the pCRIITOPO vector (*invitrogene*) and transformed them into four different *E. coli* strains. Surprisingly, while PCR products from wild-type telomeres were easily cloned into those *E. coli* strains, clones from telomeres of type II survivors were always difficult to get. The rarely recovered cloned contained inserts between 0 and 400 bp, which are smaller than the sizes of PCR products. This result either there are repetitive sequences in the telomeres of type II survivors, or the sequence itself encodes genes toxic to bacteria. Finally I cloned

these strains into a special *E. coli* strain which can stablize long repetitive sequences (STAB2 cells, GIBCO-BRL). The cloned plasmid showed the expectd sizes. Sequences of the PCR products showed only T and G signals by primer 3 and showed only A and C signals by primer 4 on sequencing gels. Four different 4-base cutters were used to rule out the possibility that mini-Y' fragments were present in the middle of $(TG_{1-3})n$ repeats. Southern blot analysis showed that while the $(TG_{1-3})n$ fragment of wild-type and type I survivors can be digested into small fragments, the $(TG_{1-3})n$ fragment of type II recombinants always stayed the same length (data no shown). This result suggests that the long extended telomere fragments in of type II survivors are all $(TG_{1-3})n$.

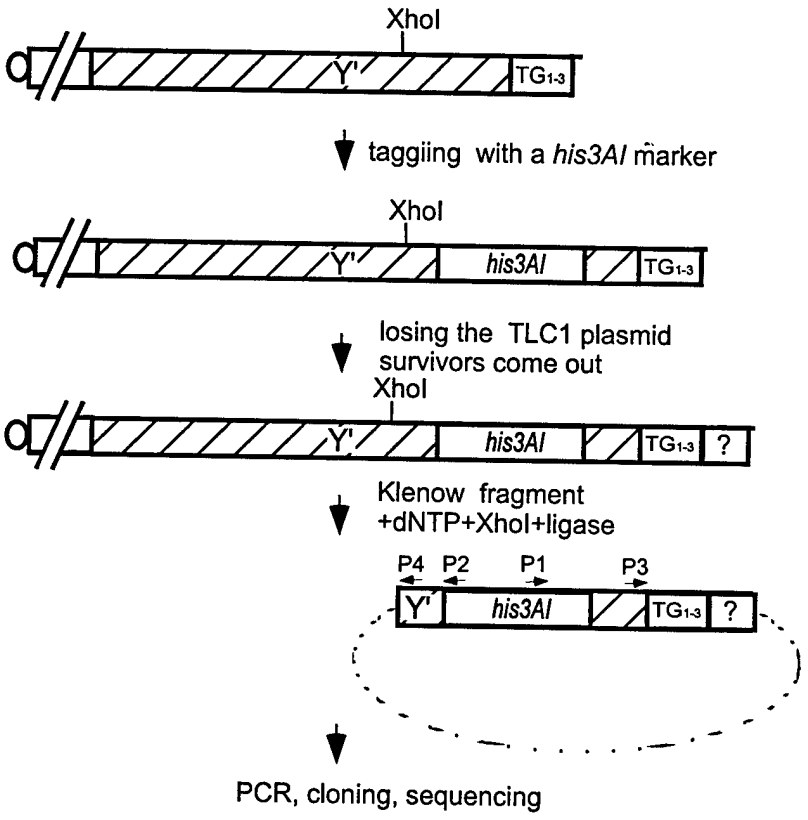

Figure 4. Scheme of cloning telomeres from type II survivors.

Recommendations in relation to the statement of work outlined in the proposal.

For the first project, I intend to optimize the condition of immuno-precipitation the *EST2* strain with nine copies of Myc tag on both the chromosomal copy and a 2 micron plasmid, and to see if I can see telomerases access to telomeres in a cell-cycle regulated mammer. For the second project, after submitting a paper to report the finding on telomere-telomere recombination, I plan to conduct a differential display experiment to look for genes been up-regulated in the *tlc1* strain during the survivor formation.

Conclusions

In the first project of understanding the nature of the *S. cerevisiae* telomerase. I found telomerase in vivo form a 20S complex. This complex contains both the RNA template and the reverse transcriptase. I am working on the chromatin IP technique to see if I can see telomerases access to telomeres in a cell-cycle regulated mammer.

In the second project I tagged the subtelomere Y' element with a marker to study telomere-telomere recombination. I found in the telomerase knockout strain, that cells can survive through telomere-telomere recombination using either TG-rich sequence or Y' sequences as substrates. I am writing a paper to report this finding now.

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Appendices

* not available